

Article

Validation of a Novel Xeno-Free Method for Human Endometrial Mesenchymal Stromal Cells (E-MSCs) Isolation and Culture

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Abstract: The cyclic regeneration of human endometrium is guaranteed by the proliferative capacity of Endometrial Mesenchymal Stromal Cells (E-MSCs). Due to this, the autologous infusion of E-MSCs has been proposed to support endometrial growth in a wide range of gynecological diseases. We aimed to compare two different endometrial sampling methods, the surgical curettage and the Vacuum Aspiration Biopsy Random Assay, and to validate a novel xeno-free method to culture human E-MSCs. Six E-MSCs cell lines were isolated after a mechanical tissue homogenization and cultured using human platelet lysate. E-MSCs were characterized for the colony formation capacity, proliferative potential and multilineage differentiation. The expression of mesenchymal and stemness markers was tested by FACS analysis and Real-Time PCR, respectively. Chromosomal alterations were evaluated by karyotype analysis, whereas tumorigenic capacity and invasiveness were tested by soft agar assay. Both endometrial sampling techniques allowed to efficiently isolate and expand E-MSCs using a xeno-free method preserving their mesenchymal and stemness phenotype, proliferative potential and multi-lineage differentiation ability during the culture. No chromosomal alterations and invasive/tumorigenic capacity were observed. Herein we report the first evidence of efficient E-MSCs isolation and culture in Good Manufacturing Practice compliance conditions, suggesting Vabra endometrial sampling as alternative to surgical curettage.

Keywords: Endometrial Mesenchymal Stromal Cells, Good Manufacturing Practice (GMP), infertility, Asherman's syndrome, endometrial thickness, Human platelet lysate (HPL), endometrial sampling

1. Introduction

The human endometrium is characterized by a high proliferative potential, as it undergoes approximately 450 regenerative cycles during woman's reproductive lifespan. Some studies reported the presence, in the basalis layer, of a small proportion of endometrial stromal cells, which were shown to be physiologically involved in the cyclic endometrial regeneration after menstrual loss [1–5]. In particular, endometrial mesenchymal stromal cells (E-MSCs) are clonogenic mesenchymal-like cells [6] localized in the perivascular space of endometrial small vessels [7] and able to express pericyte markers, such as SUSD2 [8,9]. E-MSCs were previously isolated by our group both from the healthy endometrium and from peritoneal, pelvic endometriosis; when cultured, they displayed the ability to form plastic-adherent colonies with high proliferative potential, as well as the property of undergoing multi-lineage differentiation into osteoblasts, chondrocytes, adipocytes, or endothelial cells in response to specific culture conditions [10,11]. Due to their capacity of multi-lineage differentiation and their immunosuppressive properties, E-MSCs are considered suitable candidates for performing stem cell therapy [12,13]. It has been reported, in fact, that E-MSCs are capable to differentiate in vitro into endometrial epithelial and stromal cells when exposed to oestradiol-containing media [14]. In addition, some studies have suggested the ability of human E-MSCs to repair endometrial damage both in animal models and in patients with Asherman's Syndrome (AS), a pathological condition characterized by extensive endometrial disruption and intrauterine adhesions leading to hardly reversible infertility [15]. Stem cell therapy has been tested as a potential cure for severe AS. Human CD133+ bone marrow-derived stem cells (BMDSCs) were injected in a murine model of AS, and were able to induce the proliferation of endometrial vascular cells [16]. In humans, autologous CD133+ BMDSCs infusion, in conjunction with estrogenic replacement therapy, obtained enhanced endometrial angiogenesis and growth, increased volume and duration of menses, and a significant reduction of intrauterine adhesion score in patients with severe AS [17]. Stem cells for therapeutical use could also be obtained from the endometrium: Tan et al. [18] infused autologous menstrual blood-derived endometrial stromal cells (menSCs) to patients with AS, obtaining a significant increase in endometrial thickness that facilitated pregnancy, both spontaneous or after in vitro fertilization (IVF). More recently, E-MSCs spheroids transplanted in the uterus of rats with induced AS were able to restore fertility, allowing spontaneous pregnancies in which the litter size was higher than in control AS-affected rats receiving autologous bone marrow cells (Domnina et al. 2018). Taken together, these data suggest that cellular therapy with E-MSCs, might represent a promising option to treat severe endometrial defects causing infertility. In IVF, it could also be used to sustain endometrial growth in patients with recurrent implantation failure and scarce endometrial responsiveness to oestrogens. For clinical use, however, E-MSCs are considered as Advanced Therapy Medicinal Products (ATMP), and, as a consequence, must be produced in compliance with Good Manufacturing Practice (GMP) rules [19], defining the highest standards of sterility, quality control, and documentation to ensure that cultured cells are safe. In this study, our aim was to isolate and expand human E-MSCs in GMP-compliant culture using the method that was previously set up by our group for human bone marrow-derived MSCs [20]. The main characteristic of this culture procedure is that it employs inactivated Human Platelet Lysate (HPL) instead of foetal bovine serum (FBS), being free of animal-derived constituents. Moreover, we also wanted to compare the effectiveness of two different methods of endometrial sampling to obtain E-MSCs: the surgical extensive curettage in general anaesthesia and the office-based, mini-invasive Vacuum Aspiration Biopsy Random Assay (Vabra).

2. Results

2.1. Isolation of E-MSCs

Six patients with mean age 29.6 ± 7.3 years (range 22-35) were enrolled; three of them underwent endometrial curettage, and the other three Vabra procedure during hysteroscopy (Supplementary Table 1). Adherent cells were observed in all cases after 7 days of culture, and in the following 15 days (first passage) a confluent layer formed of cells with an elongated, fibroblastic shape was rapidly generated. No morphological differences in the colony shape (Figure 1 A and B) and during the expansion (Figure 1 C and D) were observed comparing Cur-E-MSC and Vab-E-MSC cell lines.

2.2 Colony formation and cellular expansion analysis

At each passage, E-MSCs showed a viability of 98%–100% in all the analysed samples, with no differences between Cur-E-MSC and Vab-E-MSC cell lines. The CFU-F number was calculated in relation to the initial cell number: Cur-E-MSCs showed a mean of 150 CFU-F per 106 cells and Vab-E-MSC a mean of 126.2 CFU-F per 106 cells, not significantly different. The cumulative PD after three passages was 11.5 ± 1.0 for the Cur-E-MSCs and 9.0 ± 3.8 for the Vab-E-MSCs, whereas after six passages it was 21.5 ± 3.9 and 20.5 ± 3.0 , respectively, showing no significant difference (Figure 2).

2.3 Cytofluorimetric analysis

At each passage cells were analysed for their expression of mesenchymal, hemopoietic and endothelial markers: overall, Cur-E-MSCs and Vab-E-MSCs expressed comparable levels of all tested markers (Table 1). The presence of epithelial, endothelial and hematopoietic cell contamination was excluded by the absence of EPCAM, CD31, CD45, CD19, and HLA-DR (Figure 3). No significant difference was observed in the marker expression at the first, third and sixth cell passage between Cur-E-MSCs (Figure 3A and C) and Vab-E-MSC (Figure 3B and D). These data indicate that the two E-MSC cell lines had a similar mesenchymal phenotype, steadily maintained during in-vitro culture.

2.4 Stemness evaluation

The mRNA expression of the stemness-related genes Homeobox protein (NANOG), octamer-binding transcription factor 4 (OCT4), and SRY (sex determining region Y)-box 2 (SOX2) were analysed by Real Time PCR. No significant differences between Cur-E-MSCs and Vab-E-MSCs cell lines were observed in the overall mean gene expression (Figure 4A) and in gene expression at different cell passages (Figure 4B and C), showing that both cell lines displayed similar stemness phenotype, steadily maintained during the in-vitro culture.

2.5 In Vitro Differentiation

At the third passage, cells of both studied lines were induced to differentiate into different lineages using specific media. After osteogenic differentiation, cells of both lines contained crystals of calcium ossalate (Figure 5A and D). Both cell lines generated adipocytes, revealed as round cells accumulating intracytoplasmic lipid vacuoles, positive at Oil Red O staining (Figure 5B and E), and chondrocytes, appearing as cells aggregated in spheres positive to Alcian Blue stain of hyaluronic acid and sialomucin (Figure 5C and E). Overall, both Cur-E-MSCs and Vab-E-MSCs cell lines showed comparable multilineage differentiation in appropriate culture conditions.

2.6 Karyotype Analysis

Cur-E-MSCs and Vab-E-MSCs cell lines were analysed at the first, third and sixth passage: all karyotypes were reported as normal (46, XX) and no chromosomal alterations (translocations, deletion/additions or aneuploidies) were noticed, showing no detrimental effect of in-vitro culture on chromosomal stability (Figure 6A and B).

2.7 Invasion assay

Both Cur-E-MSCs and Vab-E-MSCs cell lines did not show invasive capacity and tumorigenic activity in the Soft Agar culture assay (Figure 6C and 6D); differently, the Sjsa positive control (osteosarcoma cell line), clearly formed colonies after 21 days in the same conditions (Figure 6E).

2.2. Figures, Tables and Schemes

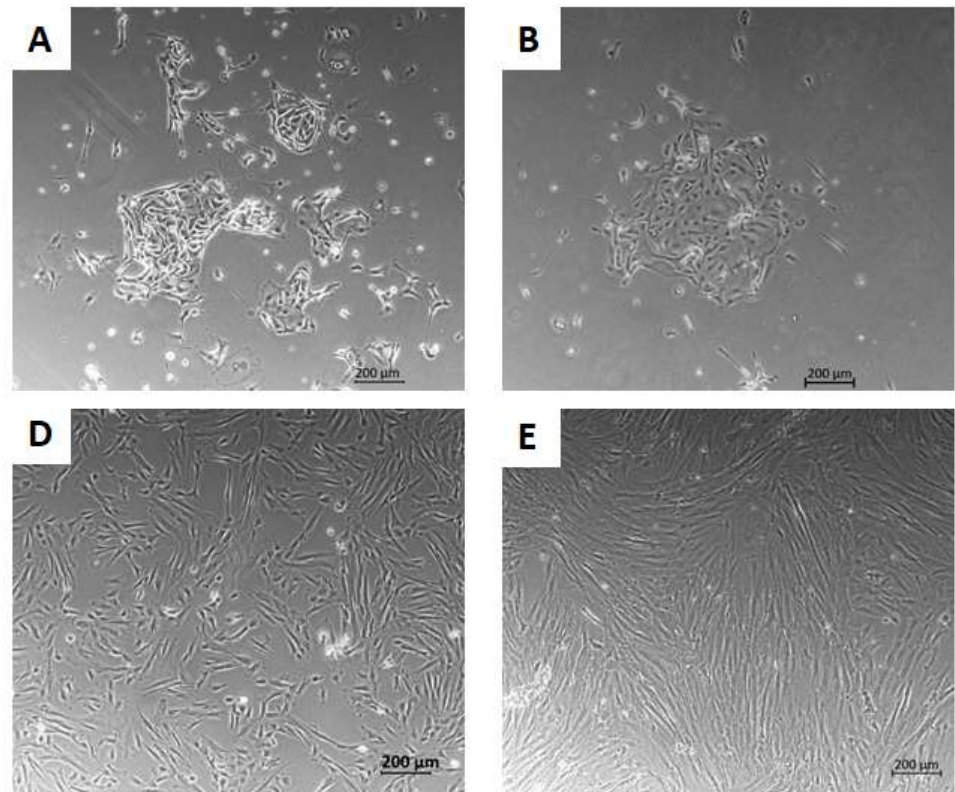


Figure 1. Morphology of E-MSCs obtained from curette and vabra biopsies. Representative phase images at ×5 magnification of CFU-Fs after 4 days from seeding and of sub-confluent cell culture during the expansion at P5, respectively in Cur-E-MSCs (A and C) and Vab-E-MSCs (B and D)

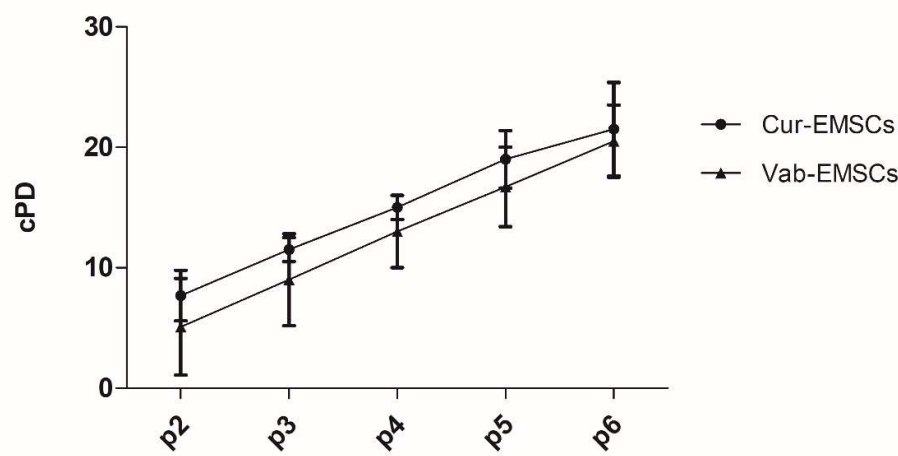


Figure 2. Growth kinetic curves of Cur-E-MSCs and Vab-E-MSCs during the expansion. Cellular growth is expressed as mean number of cumulative PD as a function of time, from the first to the sixth passage (p1-p6). The two cell lines showed no significant differences.

Table 1. Cytofluorimetric evaluation of surface marker expression of Cur-E-MSCs and Vab-E-MSCs . Quantitative mean expression of mesenchymal, hematopoietic and endothelial markers was assessed by FACS analysis. Values represent the percentage of positive cells and are expressed as mean \pm SD. The immunophenotype of both E-MSC cell lines showed no significant difference during the culture.

	Cur-E-MSCs (n=3) (%)	Vab-E-MSCs (n=3) (%)	<i>p value</i>
CD90	97.2 \pm 2.7	93.2 \pm 7.9	<i>ns</i>
CD73	97.5 \pm 4.7	99.2 \pm 0.8	<i>ns</i>
CD105	82.2 \pm 20.5	70.3 \pm 27.3	<i>ns</i>
CD146	80.6 \pm 27.6	62.8 \pm 20.2	<i>ns</i>
SUSD2	30.5 \pm 9.8	32.0 \pm 24.7	<i>ns</i>
CD45	0.6 \pm 0.5	2.2 \pm 0.7	<i>ns</i>
CD29	0.6 \pm 0.2	0.7 \pm 0.4	<i>ns</i>
HLA-DR	0.3 \pm 0.3	0.3 \pm 0.4	<i>ns</i>
CD31	2.2 \pm 2.5	2.8 \pm 2.0	<i>ns</i>
EPCAM	0.3 \pm 0.3	0.5 \pm 0.4	<i>ns</i>

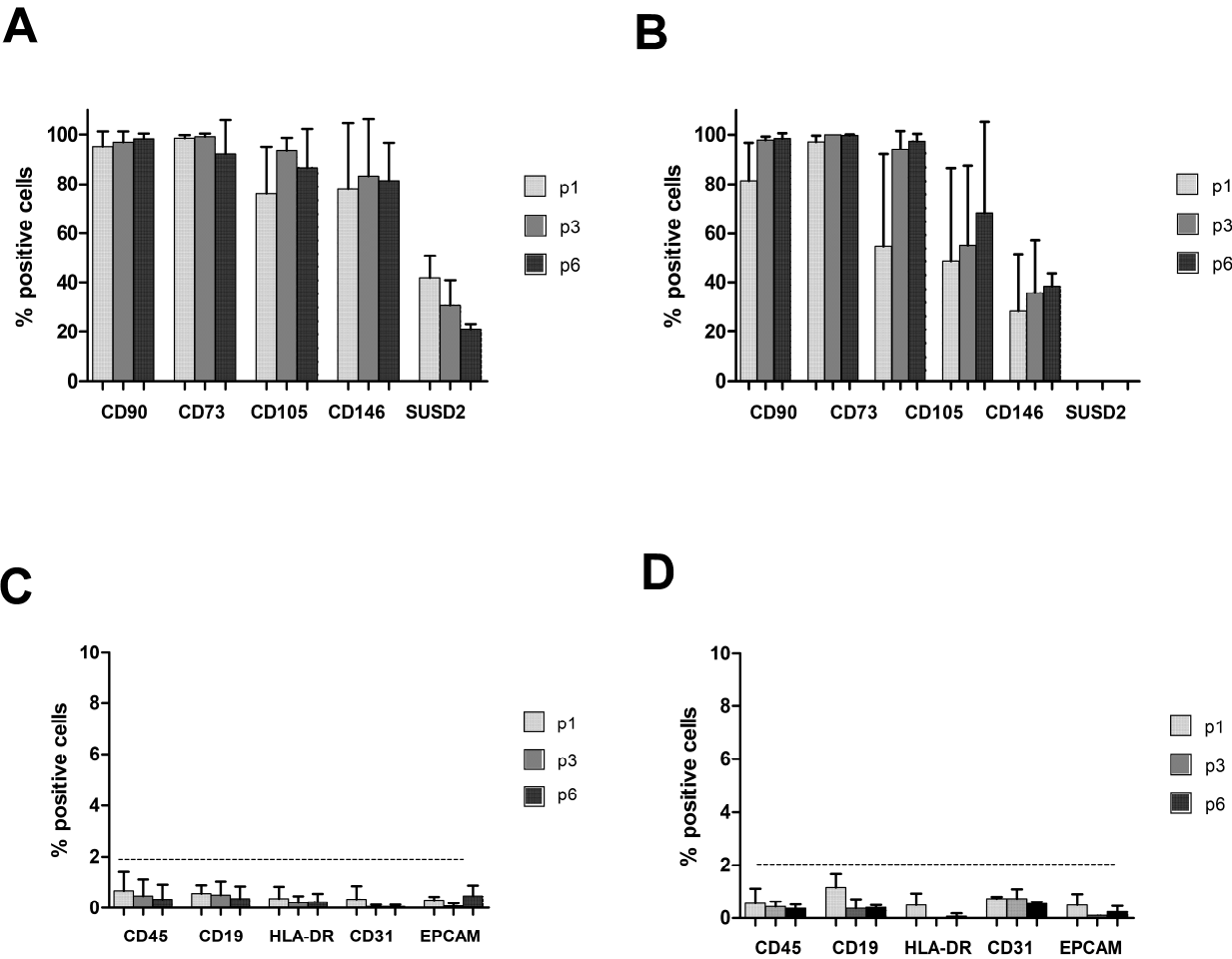


Figure 3. Flow cytometry analysis of mesenchymal, hematopoietic and epithelial markers of Cur-E-MSCs and Vab-E-MSCs at the first, third and sixth passage. Histograms show marker expression in Cur-E-MSCs (A and C) and Vab-E-MSCs (B and D) during the expansion at the first (p1), third (p3) and sixth (p6) cell passage. No differences of immunophenotype were observed during the expansion of the two cell lines.

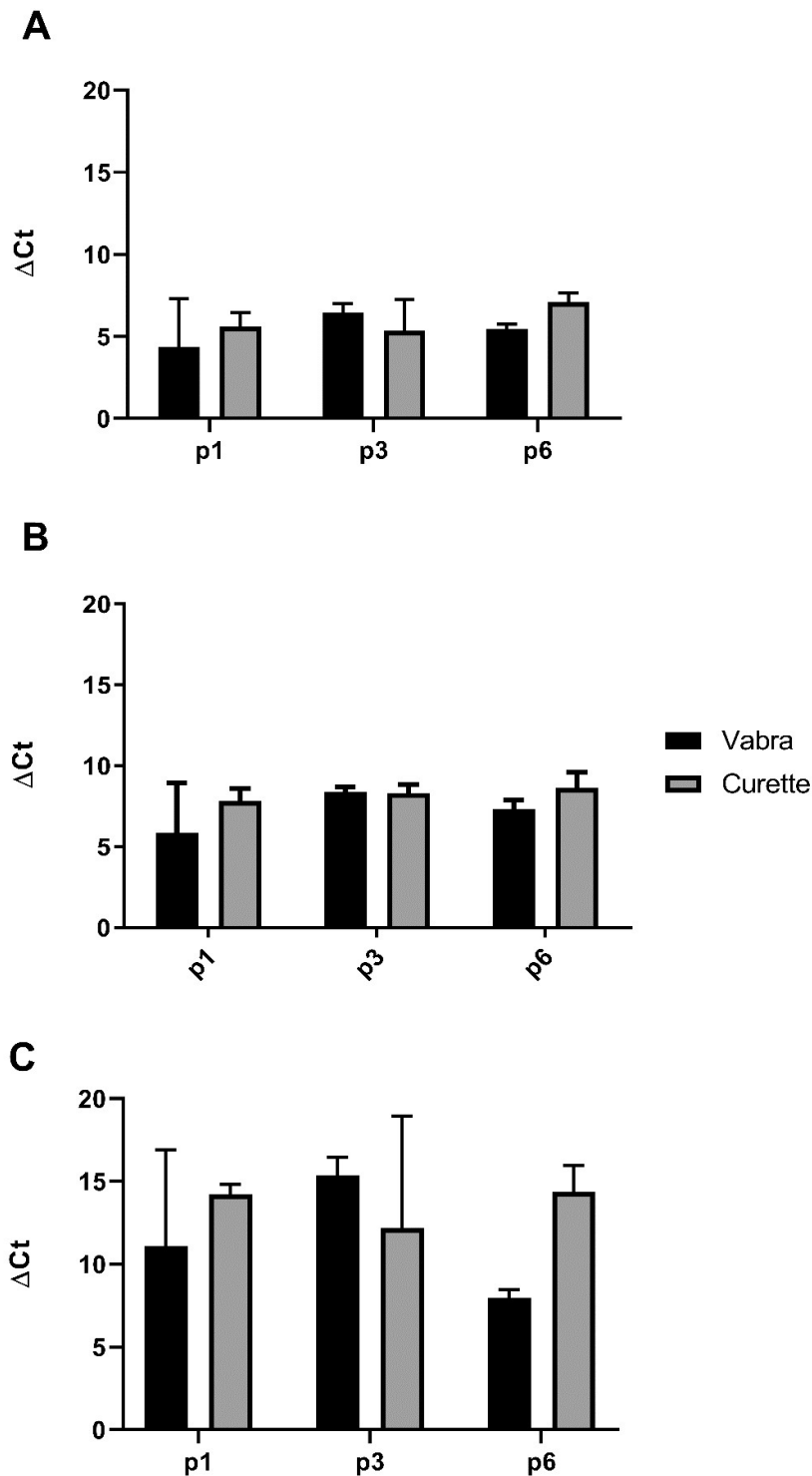


Figure 4. mRNA expression of NANOG, OCT4 and SOX2 in Cur-E-MSCs and Vab-E-MSCs during the expansion. Mean expression of NANOG (A), OCT4 (B) and SOX2 (C) in Cur-E-MSCs and Vab-E-MSCs cell lines at the first (p1), third (p3) and sixth (p6) cell passage. Data were expressed as ΔCt values calculating as [CT target gene- CT housekeeping gene] during the expansion

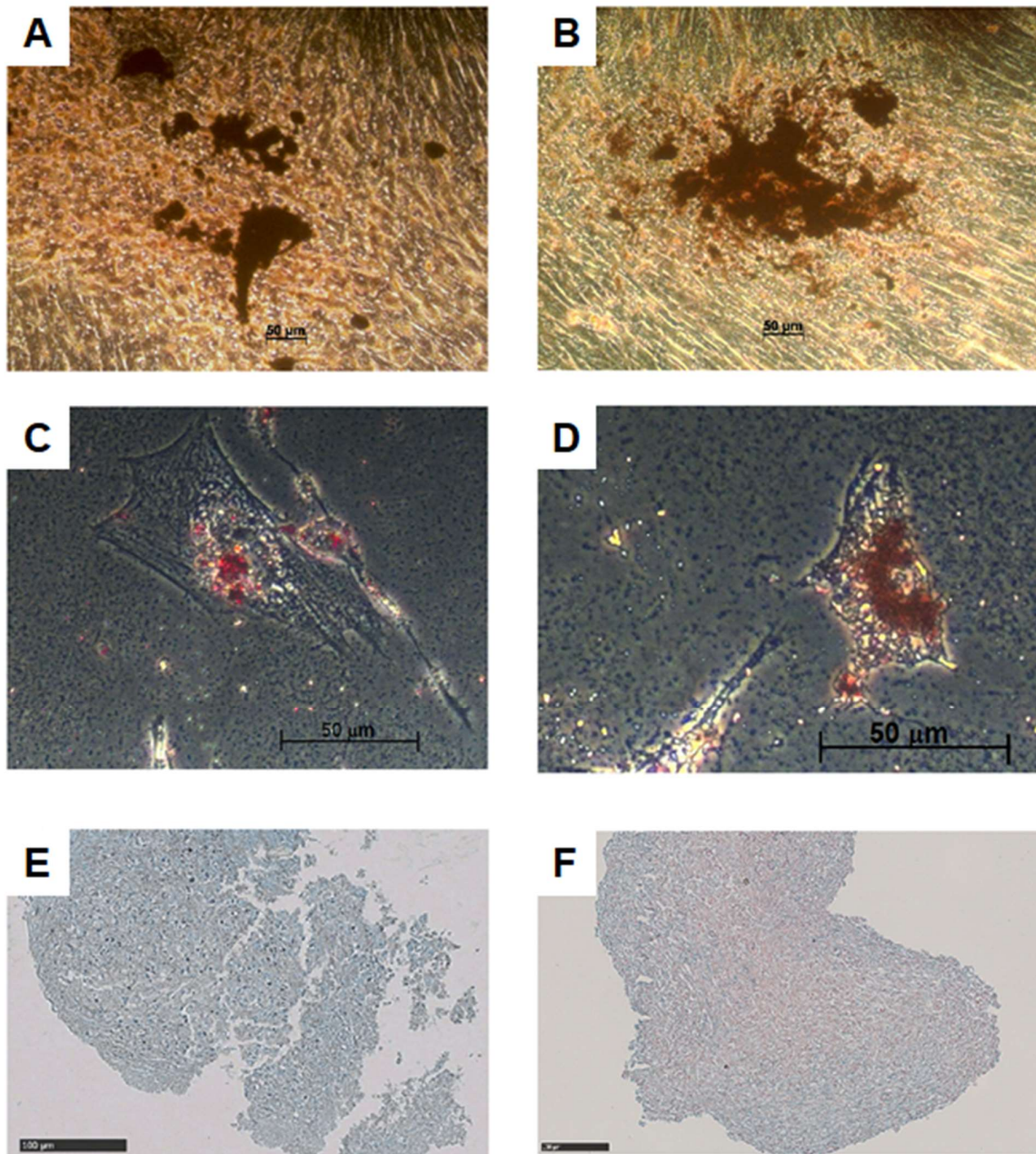


Figure 5. Differentiation potential assay after 3 weeks of specific induction (three different conditions). Induced differentiation in Cur-E-MSCs (left column), and Vab-E-MSCs (right column). Von Kossa staining (A and D, magnification 20X) shows the presence of calcium oxalates, indicating osteogenic differentiation. Oil red O stain (B and E, magnification 40X) shows intra-cytoplasmic vacuoles, indicating differentiation into adipocytes. Alcian blue stain (C and F, magnification 20X) shows the hyaluronic acid content, typical of chondrocytes.

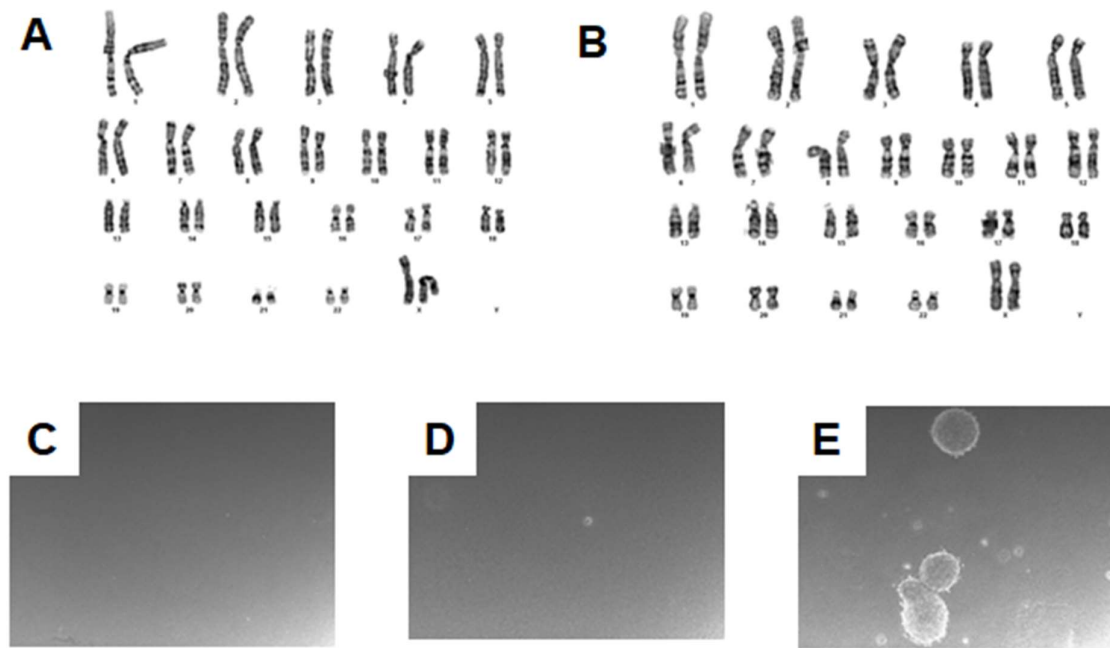


Figure 6. Karyotype analysis and soft agar assay to assess genetic modification and/or tumorigenesis potential in Cur-E-MSCs and in Vab-E-MSCs. Normal karyotype at the 6th passage (A and B), showing no genetic alteration during the expansion of either Cur-E-MSCs (left) and Vab-E-MSCs (right). No colonies were observed in either Cur-E-MSCs or Vab-E-MSCs in soft agar assay (C and D); in (E) primary osteosarcoma cells used as positive control.

3. Discussion

It is rather well established that when endometrial thickness does not reach a peak value of 7 mm in the ovulatory phase of the cycle the occurrence of pregnancy, either spontaneous or after transfer of an in vitro-produced embryo, is significantly less likely [21,22]. When endometrial growth is inadequate, estrogen administration during the menstrual cycle is frequently the chosen treatment, yet this therapeutic approach is efficient only in a limited number of patients, due to the rather frequently observed limited responsiveness of the tissue to ovarian hormones [23]. The extreme form of endometrial impairment is Asherman's syndrome (AS)[15]. Current treatments for AS include hysteroscopic adhesiolysis followed by antibiotic and hormonal therapy aimed at avoiding recurrent adhesive disease; however, they are poorly effective in restoring endometrial growth and function [24]. As epithelial and stromal cells with stem cell characteristics are found in the basalis layer of the human endometrium, where they were shown to be involved in the cyclic endometrial regeneration [1–4], it has been hypothesized that a thin endometrium which is refractory to estrogens could be the result of a defective number or function of endometrial stromal cells. Following this idea, the infusion of autologous E-MSCs could represent a novel therapy for endometrial regeneration and growth enhancement [25,26]. According to cell therapy regulation, E-MSCs for human clinical application must be isolated and produced in GMP conditions using standardized techniques. In the present study, we aimed at validating a xeno-free method GMP compliant method to isolate and expand E-MSCs, that was previously set by our group for human bone marrow-derived MSCs [20,27]. In order to avoid the use of digestive enzymes and FBS, we set up the isolation procedure with mechanical digestion to homogenate the endometrial samples, and then we cultured the cells with HPL addition instead of FBS, obtaining a medium devoid of animal components. We showed herein that this culture technique can be successfully applied to isolate and expand human E-MSCs. In fact, our E-MSCs showed remarkable adherent properties, colony formation

and proliferative potential, as well as mesenchymal stromal characteristics matching the criteria of the International Society for Cellular Stem Cells International Therapy (ISCT) [28]. The cultured E-MSCs showed stemness characteristics and mesenchymal phenotype, that were steadily maintained during the in-vitro culture up to sixth passages, as well as differentiation potential into chondrocytes, adipocytes and osteoblasts. Moreover, no chromosomal alterations were observed in all expanded batches, suggesting no detrimental effects of the prolonged culture on chromosomal stability. Finally, they did not show invasive and growth capacity in soft agar, confirming the complete safety of this population for clinical use. Overall, quality controls needed to guarantee the safety for clinical administration of these E-MSCs to patients resulted compliant at the batch release as required by the GMP regulations [19,29,30], supporting their potential clinical use to enhance endometrial growth in case of estrogen-resistant endometrium or in AS. We also compared the efficiency of two endometrial sampling methods, the surgical curettage, previously used by our group to get MSCs from the endometrium and from both ovarian and peritoneal endometriosis [10] and the Vacuum Aspiration Biopsy Random Assay (Vabra), a less invasive, cheap office-based method not requiring anesthesia. Herein we showed that isolation and expansion of E-MSCs was equally efficient starting from samples obtained by either biopsy method, and that the cell characteristics were not influenced by the endometrial sampling technique. Notably, Vabra allowed the recovery of a reduced amount of endometrial tissue compared to the surgical curettage, requiring a prolonged in vitro culture in the very first cell passage to obtain an adequate cell population for further experiments. However, as previously described, the proliferation rate in the subsequent cell passages resulted compared between the two populations. In addition, Vabra sampling was performed in patients with suspected endometrial polyps, requiring further laboratory precautions, such as strictly selective culture media and an accurate karyotype evaluation, in order to exclude the pivotal contamination of cancer cells.

4. Materials and Methods

4.1 Patients

E-MSCs were taken from healthy endometrial samples obtained from six patients enrolled at the Department of Surgical Sciences, University of Torino, between March 2018 and June 2019. The study was conducted after approval by the Ethics Review Board (Prot. N° 0055438, May 28th 2018) and preoperative written informed consent was obtained from each patient. Endometrial sampling was performed either by surgical curettage from patients operated for benign ovarian cysts or hydrosalpinx, in general anaesthesia, or by the Vabra procedure, performed during diagnostic hysteroscopy without the need of any anaesthesia (Du et al. 2016).

4.2 E-MSC isolation and culture

The obtained E-MSC cell lines were distinguished, according to the sampling procedure, as Cur-E-MSCs (curettage-derived) and Vab-E-MSCs (Vabra-derived). Endometrial samples were collected in a sterile tube containing α -Mem (Sigma Aldrich, St. Louis) with 1% L-glutamine (Gibco, USA), 1% penicillin/streptomycin (Sigma Aldrich) and 10% HPL (Human Platelet Lysate), prepared by the "Production and Emocomponent Validation Centre" of Turin as previously described [20] and mechanically homogenised using the MACSmix™ Tube Rotator in combination with "Mito Tissue" program of the Dissociator GentleMacs without using digestion enzymes (Miltenyi Biotec, Germany). Cells were seeded at a density of 10,000 cells/ cm² in the same medium of the collection in six-well multiwell plates for CFU evaluations and in T75 culture flasks (d). After 7 days, detached cells were discarded, whereas the adherent cells were refed every 2-3 days. After reaching confluence, which was achieved usually 10 days after seeding, cells were detached using Trypsin (Sigma Aldrich) and re-plated at a density of 1,000 cells/ cm² for 6 passages as maximum. A sterility test (Bact Alert; Bioré) was performed on the endometrial samples before seeding in order to exclude bacterial or fungal

contamination At each passage, the cultured cells were analysed for growth, viability, immuno-phenotype, RNA expression of stem cells markers and chromosomal stability. The differentiative potential and invasion assay were evaluated at the third culture passage.

4.3 Colony formation assay, cell viability and proliferative capacity

To evaluate the clonogenic potential, fibroblastic-colony-forming unit (CFU-F) assay was per-formed for each cell batch. After homogenization, cells were seeded in duplicate in six-well plates at a density of 10,000; after 7 days from seeding, they were fixed in acetone/methanol (1:1) and stained with the May Grunwald Giemsa dye (Sigma Aldrich) to allow cell count. Cell count and viability assay of the cells before seeding were performed using Burker chamber after treatment with Turk's liquid to lyse the red blood cells and 1:1 Trypan Blue staining. Clusters including more than 50 cells were considered colonies. The CFU-F value was calculated as mean of the number of colonies obtained after seeding and expressed as number of CFU-F frequency in 10⁶ seeded cells. The cell proliferative capacity during expansion was expressed as Population Doubling (PD) using the following formula: $\text{Log}_{10}N / \text{Log}_{10}2$, where N was the cell number of the detached cells di-vided by the initial number of seeded cells. The cellular expansion growth was expressed as cumulative PD (cPD) as previously described (Mareschi et al. 2006).

4.4 Flow cytometric analysis

Cell suspension (1-2 x 10⁶) was incubated with antibodies for 20 min at 4°C in 100µl of phosphate buffered saline (PBS). The following anti-human monoclonal antibodies, all fluorescein isothio-cyanate (FITC)- or phycoerythrin (PE)- or allophycocyanin (APC)-conjugated, were used at 1:10 dilution: anti-CD90 FITC (ref: IM1839U), anti-CD73 PE (ref: B68176), anti-CD105 PC7 (ref: B43293), anti-CD45 FITC (ref: IM0647), anti-CD34 FITC (ref: IM1870), anti-CD14 FITC (ref: B36297), anti-HLA-DR PE (ref: IM1639), anti-CD19 APC (ref: IM2470), anti-CD31 FITC (ref: IM1431U) (Beckman Coulter, Brea, CA, USA), anti-CD146 APC (clone: REA773), anti-SUSD2 APC (clone: W5C5), and anti-EPCAM FITC (clone: HEA-125) (Miltenyi Biotech, Germany). As negative control, cells were incubated without antibodies. Labelled cells were washed with PBS and ana-lysed using Navios cytometer (Beckman Coulter).

4.5 Real-time PCR analysis

Total RNA extraction was performed using the Maxwell automatic extractor (Promega, Madison, WI) according to the manufacturer's protocol, and the obtained RNA was quantified by Nanodrop (Thermo Scientific, Wilmington, DE, USA). First-strand cDNA was produced from 1µg of total RNA using the GeneAmp 9700 Thermal Cycle (Applied Biosystems, Foster City, CA, USA). Re-al-time PCR experiments were performed in a 20µl reaction mixture containing 100ng cDNA template and primers designed by Primer Express™ version 3.0 (Applied Biosystems, Foster City, USA). Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) mRNA was used to nor-malize RNA inputs. Gene expression was performed for the following markers: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-CAAGGTCATCCATGACAAC-3', 5'-GTGGCAGTGATGGCATGGAC-3'; Homeobox protein (NANOG), 5'-GCCAGGGGTCTCGATCTC-3', 5'-GGTGGCTCACGCCTGTAAAT-3'; octamer-binding tran-scription factor 4 (OCT4), 5'-ACCCACACTGCAGCAGATCA-3', 5'-CACACTCGGACCACATCCTTCT-3'; SRY (sex determining region Y)-box 2 (SOX2), 5'-TGCGAGCGCTGCACAT-3', 5'-GCAGCGTGACTTATCCTTCTT-3'. A respective amount of 500 nmol of specific primers and 200 nmol of specific probe was used. Relative quantification of the products was performed using a 96-wells plate with the Taqman enzyme amplification pro-cess (ABI PRISM 7500 real time system, Life technologies, Texas, USA). Thermal cycling condi-tions were as follows: activation of GoTaq® qPCR Master Mix (Promega) at 95°C for 2 min, fol-lowed by 40 cycles of amplification at 95°C for 15 seconds and 60°C for 1 min. We compared the expression of target gene in the different batches using ΔCt values calculating as [CT target gene- CT housekeeping gene] during the expansion.

4.6 Karyotype analysis

Karyotype analysis was performed after cells were arrested at metaphase by incubation with Colcemid (Invitrogen Corporation, Grand Island, NY, USA), maintained in a hypotonic solution (0,075 M KCl), fixed with methanol/acetic acid 3:1 (Merck, Milan; Italy), and stained with Giemsa using standard laboratory protocols. At least 20 metaphases were analysed using MackType software (Nikon Corporation, Japan) according to the International System for Human Cytogenetic Nomenclature.

4.7 Differentiation Potential Assay

For differentiation experiments, E-MSCs were cultured at the third passage in osteogenic, adipogenic and chondrogenic medium according to the manufacturer's instructions. Briefly, for osteogenic induction 45,000 cells were plated in each well of six well plates and cultivated in StemMACS OsteoDiff Media (Miltenyi, Germany): after 21 days, osteogenic differentiation was demonstrated by the accumulation of calcium (crystalline hydroxyapatite detection by Von Kossa staining). For adipogenic differentiation, 75,000 cells were cultured in StemMACS AdipoDiff Media differentiation medium (Miltenyi, Germany) for 21 days, after which the presence of intracellular lipid vesicles was assessed after fixation with paraformaldehyde vapours and Oil Red O staining. For chondrogenic differentiation, an aliquot of 250,000 cells were cultured in StemMACS ChondroDiff Media differentiation medium (Miltenyi, Germany) for 21 days in 15 mL polypropylene culture tubes; during chondrogenic differentiation, cellular growth occurred as cellular aggregates floating freely in suspension, the pellet was included in paraffin and stained with Alcian Blue to identify the presence of hyaluronic acid and sialomucin.

4.8 Soft agar assay

To exclude the tumorigenic capacity and invasiveness of E-MSCs, the soft agar assay was performed using a commercial tumour mesenchymal cell line (Sjsa) as a positive control. A 4% solution of noble agar (Becton, Dickson and Company, Le Port de Claix, France) was prepared as previously described in The Soft Agar Colony Formation Assay (Borowicz et al. 2014) (The E-MSCs at P3 in each culture condition were harvested, washed and seeded at a final density of 5000 cells/well in 6-well plates in duplicate. The test was performed with the use of 0.8% and 0.4% agar in Alpha Mem added with 1% of L-Glutamine, 1% of Pen/Strep and 10% of HPL, arranged, respectively, at the base and on the surface. The cells were incubated for 21 days at 37°C in the presence of 5% CO₂ and 100 µl of culture medium were added twice a week. After incubation, the colonies were counted with the use of the inverted microscope.

4.9 Statistical analysis

Results were analysed by GraphPad Prism V7 software (GraphPad Software Inc., San Diego, CA, USA). Data were analysed for normality using the Shapiro-Wilk test, and then expressed as mean ± SD. Differences in mRNA expression level and in the immunophenotype among groups were investigated using 2-way Anova with Bonferroni's multiple comparison test. Significance was set at $p < 0.05$.

5. Conclusions

In conclusion, herein we report the validation of a new GMP-compliant method to isolate E-MSCs from human endometrium, as well as the comparison between cells obtained by two different sampling techniques, surgical curettage and Vabra. The novel culture method proposed herein is characterized by a mechanical digestion (instead of enzymatic digestion) of the biopsied sample and by the use of inactivated HPL (instead of FBS) that avoids the presence of animal-derived products thus preventing the risk of transmitting infectious agents and of eliciting immunization. The E-MSCs isolated and expanded by this culture method showed all the characteristics of MSCs as defined by the ISCT, preserving the stemness markers and showing no karyotype modification or tumorigenic potential. Our observations suggest that: (a) the novel culture technique

proposed herein guarantees a safe and efficient GMP-compliant method to isolate E-MSCs for future clinical application, and (b) that a simpler, cheaper and quicker endometrial sampling technique like Vabra can be successfully used to obtain E-MSCs instead of the surgical curettage in general an-aesthesia.⁶ Patents

Supplementary Materials: Supplementary Table 1: Clinical characteristics of patients enrolled for the study.

Curette cell lines			
Cell line	Age	Indication	Other pathologies
E-MS01	22	Benign ovarian cyst	none
E-MS05	29	Benign ovarian cyst	none
E-MS09	33	Benign ovarian cyst	none
Vabra cell lines			
Cell line	Age	Indication	Other pathologies
E-MS02	35	Endometrial polyp	none
E-MS04	33	Endometrial polyp	none
E-MS07	35	Endometrial polyp	none

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data generated or analyzed during this study are included in this published article. However, the data obtained in this study are available from the corresponding author upon request.

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